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Stimulation of Nuclear Volume Enlargement and Neuronal Process Growth by Estrogen in the Hypothalamic and Limbic Nuclei of the Rat

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ABSTRACT—Effects of estradiol-17β (E_2) on the growth of nuclear volume of the ventromedial nucleus (VMN), the suprachiasmatic nucleus (SCN) and the bed nucleus of the stria terminalis (STN), and the growth of neuronal process length in these nuclei were studied. Male and female rats of the Wistar/Tw strain were castrated on the day of birth (Day 1), and were given subcutaneous injections of 10 μg E_2 daily for the first 10 postnatal days. They were killed on Days 11 and 31. E_2 treatment generally stimulated the growth of the nuclear volume and the neuronal process length in the VMN. However, the E_2 effect was not significant in the nuclear volume of the VMN in males on Day 31 and in the neuronal process length in females on Day 11. In the SCN, E_2 failed to stimulate the nuclear volume enlargement, but it was effective for the neuronal process elongation only on Day 31. In the STN, E_2 stimulated the growth of nuclear volume on Day 31 in males and on Day 11 in females and the neuronal process length on Day 11 in males. The present results showed that the E_2 effects were marked in the VMN but were less evident in the SCN and STN, and that the E_2 response in the nuclear volume in a nucleus was generally accompanied by a similar stimulatory effect of E_2 in the neuronal process growth in the nucleus, indicating that the nuclear volume enlargement is due, at least partly, to the neuronal process growth.

INTRODUCTION

Sexual dimorphism has been demonstrated in some discrete areas of the brain, such as an intensely staining area of the medial preoptic area, called the sexually dimorphic nucleus of the preoptic area (SDN-POA) [1–3], the ventromedial nucleus (VMN) [4], the medial nucleus of the amygdala [5] and the medial preoptic nucleus (MPN) in the rostroventral periventricular region of the POA [6, 7]. The volumes of the SDN-POA, VMN and amygdala nuclei in male rats were greater than those in females, and these nuclei in females grew to the male level by perinatal treatment with sex steroids [1, 3–5]. On the other hand, Ito *et al.* [7] reported that the volume of the MPN in female rats was greater than that of males and decreased to the male level by neonatal steroid treatment.

These morphological sexual dimorphisms in the nuclear volume may be due to the differences in individual neuronal size, total number of neurons and/or neuronal density of the nuclei, which are induced by exposure to testicular androgen during the perinatal period. With regard to the sex steroidal influence on the neurogenesis for the sexual differentiation of the brain, Toran-Allerand [8, 9] studied neuronal process outgrowth in newborn mouse hypothalamus-POA in organotypic culture, and this was confirmed by our studies in monolayer culture of cells derived from the hypothalamus-POA of neonatal mice, fetal and neonatal rats [10–12]. *In vivo* studies also substantiated the findings of these *in vitro* effects of sex steroids. Hammer and Jacobson [13] reported that the dendritic extent and neuronal size in the SDN-POA were found to increase more in male rats than females during the first 10 postnatal days. Recently we found that subcutaneous injections of estradiol-17β (E_2) for the first 10 postnatal days in both sexes of rats castrated on the day of birth increased both the nuclear volume and the total

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process length of SDN-POA neurons at 11 and 31 days of age.

To afford further evidence for the steroidal effects on the development of sexual dimorphism in the rat brain, the effects of E_2 on the growth of nuclear volume and neuronal process length were quantitatively studied in the VMN, the suprachiasmatic nucleus (SCN) and the bed nucleus of the stria terminalis (STN).

MATERIALS AND METHODS

Male and female rats of the Wistar/Tw strain were castrated on the day of birth (Day 1) under hypothermal anesthesia. Subsequently they were subcutaneously injected with 10 μg E_2 (Sigma) dissolved in 0.02 ml sesame oil daily for the first 10 postnatal days. As controls castrated rats were given injections of 0.02 ml vehicle oil only. In the study of neuronal process length, sham-operated controls were also prepared. On Day 11 or 31 rats were subjected to intracardiac perfusion under ether anesthesia.

For the study of nuclear volume 10% formalin was used as perfusion fluid. The brains were removed and placed in 10% formalin for at least 5 days. Paraffin sections of the brains were cut frontally at 15 μm thickness, and every third sections were stained with cresyl violet (Nissl preparations). Representative profiles of the VMN, SCN and STN are shown in Figure 1. The outlines of these nuclei in both sides of the brain were traced in all the third sections with a camera lucida at $\times$ 98.5 magnification. From the drawings, the nuclear area was measured with the aid of a tablet digitizer (Mutoh Industry Ltd.), and the nuclear volume (the mean of left and right nuclei) was calculated from the measurements.

The neuronal process length was measured using Golgi-stained preparations. Rats were anesthetized with ether and perfused with 0.9% NaCl followed by freshly prepared Golgi-Hortega fixative [14] consisting of potassium dichromate (5 g), chloral hydrate (5 g), formaldehyde (5 ml), 50% glutaraldehyde (5 ml), dimethyl sulfoxide (5 drops) and deionized water (100 ml). Brains were removed and placed in a fresh Golgi-Hortega fixative, which was changed daily for 4 days.

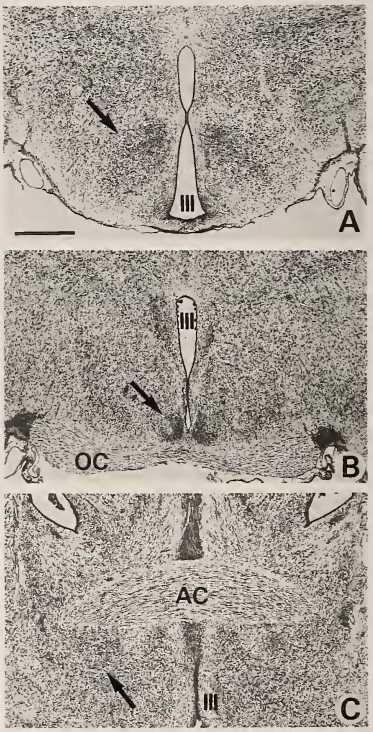

Fig. 1. Microphotographs of representative frontal sections in neonatally castrated control male rats on Day 31. Cresyl violet preparations. (A) ventromedial nucleus (VMN), (B) suprachiasmatic nucleus (SCN), (C) bed nucleus of the stria terminalis (STN). Arrows indicate these nuclei. AC, anterior commissure; OC, optic chiasma; III, third ventricle. Bar: 200 μm.

Whole brain was then stained in 0.75% $AgNO_3$ for 4 days, embedded in celloidine and frontally sectioned at 120 μm thickness. Completely impregnated neurons (Fig. 2) in both sides of the VMN, SCN and STN in sham-operated and castrated control rats and E_2-injected ones of both sexes were traced with a camera lucida at $\times$395 mag-

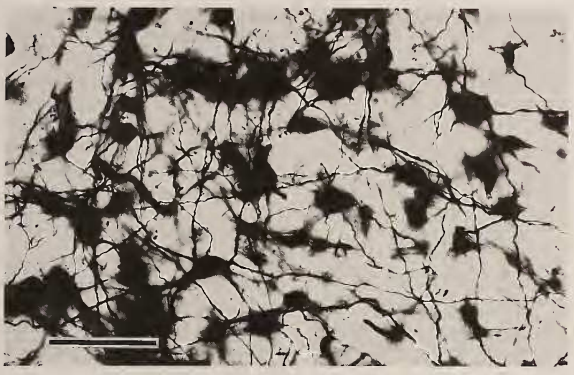

FIG. 2. A part of the VMN of an E_2-injected male rat on Day 31. Golgi-stained preparation. Bar: 100 μm.

nification. Total neuronal process length per neuron was measured using the tablet digitizer. The mean number of neurons measured per rat in the VMN, SCN and STN was 16.5, 7.8 and 17.7, respectively. As the double staining of the VMN, SCN and STN neurons by $AgNO_3$ and cresyl violet was unsuccessful, the area of measurement in a Golgi preparation was determined by referring to a separately stained Nissl preparation.

The results were analyzed by Mann-Whitney U-test.

RESULTS

Nuclear volume

Figures 3–5 show the effects of E_2 on the growth of nuclear volume of the VMN, SCN and STN. E_2 clearly increased the nuclear volume of the VMN in Day 11 rats of both sexes and in Day 31 females as compared to matched controls (all comparisons, $P<0.05$). In Day 31 males, the E_2 effect on nuclear volume enlargement was statistically not significant (Fig. 3). The nuclear volume of the SCN in E_2-treated rats tended to be greater than that of the controls, but the differences were statistically not significant (Fig. 4). The increase of the STN volume by E_2 treatment was detected in

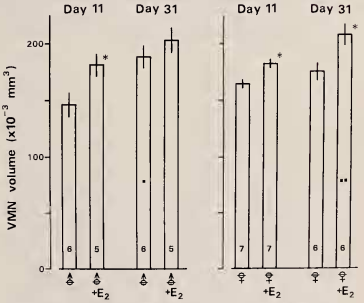

FIG. 3. Effects of estradiol-17β (E_2) on the mean nuclear volume of the VMN in Day 11 and Day 31 rats. Numbers in columns indicate the numbers of rats measured. Vertical bars depict the standard errors of means. Significance of differences: E_2-treated vs castrated control rats, *$P<0.05$; Day 11 vs comparable group of Day 31, ■$P<0.05$, ■■$P<0.01$. Significant differences between male and matched female rats were not detected.

Day 31 males and in Day 11 females (both comparisons, $P<0.05$) (Fig. 5).

Significant sex differences in the nuclear volume were rarely observed among groups. In the SCN of Day 31 E_2-injected rats the nuclear volume was

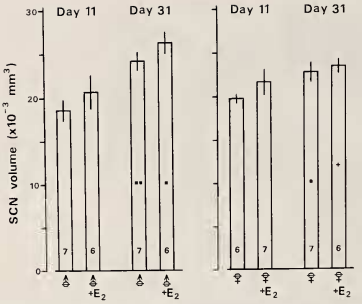

Fig. 4. Effects of E_2 on the mean nuclear volume of the SCN in Day 11 and Day 31 rats. Significance of differences: male vs matched female rats, $^{+}P<0.05$; Day 11 vs comparable group of Day 31, $^{\blacksquare}P<0.05$, $^{\blacksquare\blacksquare}P<0.01$. Significant differences between E_2-treated and castrated control rats were not detected. For other explanations, refer to legend of Fig. 3.

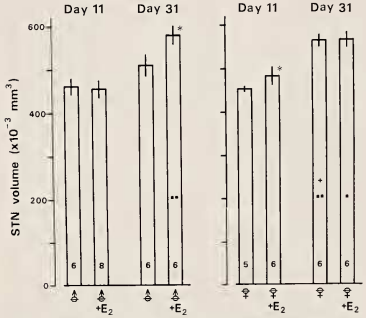

Fig. 5. Effects of E_2 on the mean nuclear volume of the STN in Day 11 and Day 31 rats. Significance of differences: E_2-treated vs castrated control rats, $^{*}P<0.05$; male vs matched female rats, $^{+}P<0.05$; Day 11 vs comparable group of Day 31, $^{\blacksquare}P<0.05$, $^{\blacksquare\blacksquare}P<0.01$. For other explanations, refer to legend of Fig. 3.

larger in males than females ($P<0.05$). While in the STN of Day 31 castrated controls it was smaller in males than females ($P<0.05$).

Age-related increase in the nuclear volume (Day 11 vs Day 31) was observed in the VMN of castrated male control rats ($P<0.05$) and E_2-injected females ($P<0.01$). In the SCN and STN the nuclear volume on Day 31 was significantly larger than that on Day 11 in the following groups: in the SCN, castrated male controls ($P<0.01$); E_2-injected males ($P<0.05$); castrated female controls ($P<0.05$), and in the STN, E_2-injected males ($P<0.01$); castrated female controls ($P<0.01$); E_2-injected females ($P<0.05$).

Neuronal process length

The effects of E_2 on the growth of neuronal process length in the VMN, SCN and STN are shown in Figures 6–8. The neuronal process length in the VMN was greater in E_2-treated male rats than castrated controls on Days 11 and 31 and was also greater in E_2-treated female rats than castrated controls on Day 31 (all comparisons, $P<0.01$). Similar stimulatory effects of E_2 were detected in the SCN in Day 31 rats of both sexes (males, $P<0.05$; females, $P<0.01$) and in the STN in Day 11 males ($P<0.05$). When E_2-treated rats were compared with sham-operated controls, the neuronal process length in the VMN was greater in the former than the latter in Days 11 and 31 females (Day 11, $P<0.01$; Day 31, $P<0.05$), but in males such stimulatory effects of E_2 were not observed. The stimulatory effects of E_2 on the SCN neurons were observed only in Day 31 females ($P<0.05$). In the STN no positive E_2 response was found when E_2-treated rats were compared with sham-operated controls.

The neuronal process length of sham-operated controls was significantly greater than that of castrated controls in the VMN of Day 11 males ($P<0.01$) and in the STN of Days 11 and 31 males (both comparisons, $P<0.05$). However, in Day 11 females the neuronal process length was smaller in sham-operated controls than castrated controls (VMN, $P<0.01$; STN, $P<0.05$). No significant differences were found in the SCN.

The comparison of the neuronal process length in the VMN between males and females showed that it was significantly greater in males than females in sham-operated control and E_2-injected groups on Day 11 ($P<0.001$ and $P<0.05$, respectively), but on Day 31 the process length in males was smaller than that in females in sham-operated

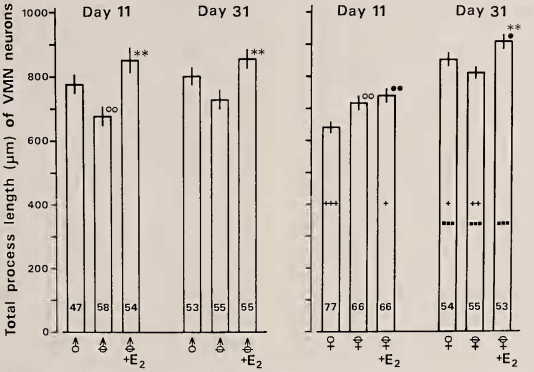

FIG. 6. Effects of E_2 on the mean total process length of neurons in the VMN in Day 11 and Day 31 rats. Numbers in columns indicate the numbers of neurons measured. Vertical bars depict the standard errors of means. Significance of differences: E_2-treated vs castrated control rats, $^{**}P<0.01$; E_2-treated vs sham-operated control rats, $^{\bullet}P<0.05$, $^{\bullet\bullet}P<0.01$; castrated vs sham-operated control rats, $^{\circ\circ}P<0.01$; male vs matched female rats, $^{+}P<0.05$, $^{++}P<0.01$, $^{+++}P<0.001$; Day 11 vs comparable group of Day 31, $^{\blacksquare\blacksquare\blacksquare}P<0.001$. ♂, ♀: sham-operated controls.

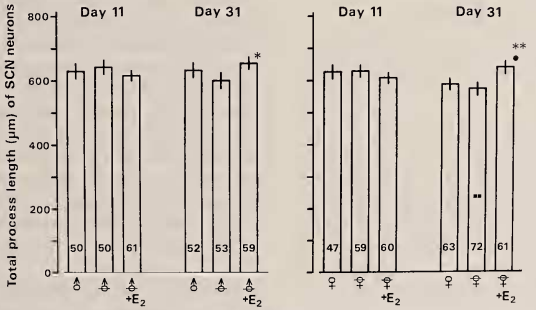

FIG. 7. Effects of E_2 on the mean total process length of neurons in the SCN in Day 11 and Day 31 rats. Significance of differences: E_2-treated vs castrated control rats, $^{*}P<0.05$, $^{**}P<0.01$; E_2-treated vs sham-operated control rats, $^{\bullet}P<0.05$; Day 11 vs comparable group of Day 31, $^{\blacksquare\blacksquare}P<0.01$. Significant differences between castrated and sham-operated control rats, and between male and matched female rats were not detected. For other explanations, refer to legend of Fig. 6.

and castrated control groups ($P<0.05$ and $P<0.01$, respectively). In the SCN sex difference was not detected. In the STN, the neuronal process length of males was significantly greater than that of females in sham-operated controls and E_2-injected rats on Day 11 ($P<0.001$ and $P<0.05$,

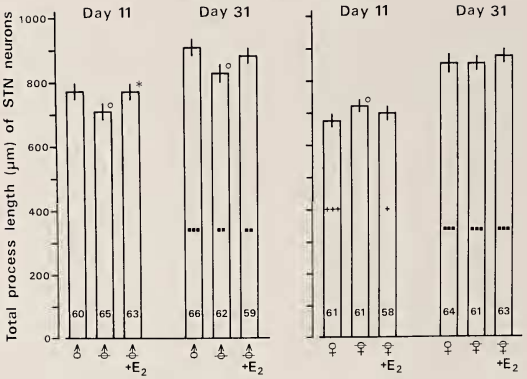

FIG. 8. Effects of E_2 on the mean total process length of neurons in the STN in Day 11 and Day 31 rats. Significance of differences: E_2-treated vs castrated control rats, $^{*}P<0.05$; castrated vs sham-operated control rats, $^{\circ}P<0.05$; male vs matched female rats, $^{+}P<0.05$, $^{+++}P<0.001$; Day 11 vs comparable group of Day 31, $^{\blacksquare\blacksquare}P<0.01$, $^{\blacksquare\blacksquare\blacksquare}P<0.001$. Significant differences between E_2-treated and sham-operated control rats were not detected. For other explanations, refer to legend of Fig. 6.

respectively). Thus, sex difference in the neuronal process length in the VMN, SCN and STN was inconsistent, likewise in the nuclear volume.

Age-related increase in the neuronal process length (Day 11 vs Day 31) was evident in the female VMN and in the STN of both sexes in sham-operated and castrated controls and E_2-injected groups (female VMN: all comparisons, $P<0.001$; male STN: sham-operated controls, $P<0.001$; castrated controls and E_2-injected groups, $P<0.01$; female STN: all comparisons, $P<0.001$). In contrast, the neuronal process length in the SCN was smaller on Day 31 than Day 11 in castrated control females ($P<0.01$).

DISCUSSION

In a previous paper we reported that neonatal injections of E_2 for 10 days clearly enhanced the growth of nuclear volume of the SDN-POA and neuronal process length in the SDN-POA in rats of both sexes [15]. These results supported the earlier findings *in vivo* [1] and *in vitro* [8–11].

The present results showing that the stimulatory effects of E_2 on the growth of nuclear volume of the VMN were apparent and the sex difference in the VMN volume of neonatally castrated control rats was not detected are in conformity with the observations by Matsumoto and Arai [4]. They reported that the volume of the VMN of normal male rats was greater than that of normal female rats and that castration on the day of birth in male rats reduced the VMN volume to a level comparable to that of normal females. They concluded that the volume of the VMN is sexually dimorphic and is modified by steriods secreted from neonatal testes.

In the SCN and STN, the E_2 response was weak and the sex difference in the volume of those nuclei was not apparent. In an earlier report Gorski *et al.* [1] found that injection of testosterone propionate to neonatal female rats or castration of neonatal males had no significant effects on the volume of the SCN, when examined in adults, and that the SCN volume of adult males castrated neonatally did not differ from that of adult females injected neonatally with sesame oil. Our present results on the SCN seem to accord with their

findings. The nuclear volume of the medial nucleus of the amygdala is greater in adult male rats than females and its differentiation occurs during the early postnatal period under the influence of sex steroids [5]. While, in the lateral nucleus of the amygdala [5], neither sex difference in the nuclear volume nor steroidal response was demonstrated. It seems likely that sex difference is detectable in brain regions which are known to control reproductive functions.

The neuronal process length in the VMN of castrated male rats was significantly less on Day 11 as compared with that of sham-operated males, and significantly increased to the level of sham-operated controls by E_2 injection. In females the length was significantly greater in E_2-injected groups than sham-operated controls. Accordingly, it is evident that the growth of neuronal process length in the VMN is enhanced by neonatal E_2 treatment. Small but significant increase of neuronal process length by E_2 injections in the SCN was observed on Day 31 but not on Day 11 in both sexes. In the STN significant increase in neuronal process length by E_2 treatment was detected only in castrated Day 11 male rats.

A certain parallelism between the neuronal process length and nuclear volume indicates that the increase in the neuronal process length by E_2 treatment is one of the factors involved in nuclear volume enlargement. However, the parallelism was not always observed. For example, the VMN volume in castrated control males was significantly larger on Day 31 than on Day 11, whereas the neuronal process length was not significantly different between Days 11 and 31. There may be some factors other than neuronal process elongation for nuclear volume enlargement.

A number of autoradiographic studies have demonstrated the accumulation of steriod hormones by neurons in the limbic and preoptic-hypothalamic structures. In adult females, a large number of neurons in the medial POA, VMN and STN were labelled with radioactive E_2 but scarcely in the SCN [16]. Testosterone-concentrating neurons were also reported in the medial POA, VMN and STN [17]. In 2-day-old female rats, Sheridan *et al.* [18] reported a topographic pattern of neuronal nuclear concentration of radioactive E_2 in these regions similar to that in the adult. Sheridan *et al.* [19] further reported that the E_2-concentrating neurons in 2-day-old female rats also concentrate testosterone. In autoradiographic study of the SDN-POA of adult rats, it was reported that for both sexes there was a greater percentage of labelled cells following E_2 exposure than following testosterone or dihydrotestosterone exposure, and the percentage of labelled cells in males following testosterone exposure was greater than that in females but such a sex difference was not found following E_2 or dihydrotestosterone exposure [20]. Our results that the nuclear volume of the SDN-POA and VMN and the neuronal process length in these nuclei markedly increased and those of the SCN showed little increase by neonatal E_2 treatment correspond well to the steroid-concentrating ability of neurons in these nuclei. However, the E_2 response of the STN failed to fit this conclusion, indicating that high steroid-concentrating ability is not always accompanied by morphological E_2 response as nuclear volume enlargement and neuronal process elongation.

ACKNOWLEDGMENTS

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